B8/B8 CIP

IN THE CANADIAN PATENT OFFICE

Examiner	:	M. Gillen
Applicant	:	Biogen, Inc.
Application No.		374,378
Filed	:	April 1, 1981
For	:	DNA SEQUENCES, RECOMBINANT DNA MOLECULES AND PROCESSES FOR PRODUCING HUMAN FIBROBLAST INTERFERON-LIKE POLYPEPTIDES

AFFIDAVIT OF WALTER C. FIERS

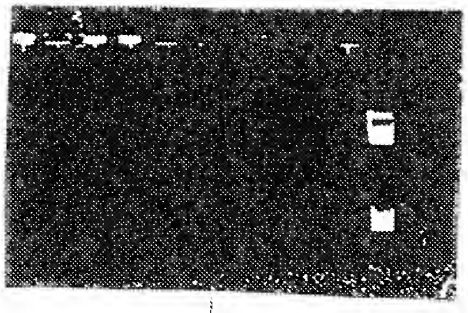
EXHIBITS 21-43

Cloning BglII fragment out SRKB67-12 in STL29

- Restriktion in K12(λ)

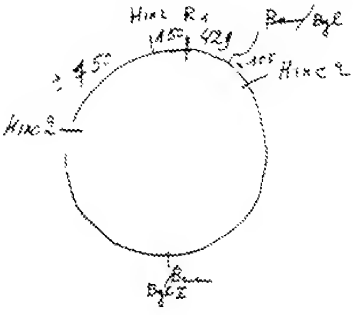
- 12 clone granatpond mit 305 zum Klebe ophagot

Restrikt HincII



Nr 8

Nr 8 left complete banden



Opened Dec 30 2002
Decachetée le
Commissioner of Patents
Commissaire des brevets
In presence of examiner
en présence de l'examinateur

1670
1750
restriktk

Plasmid ground STL29-8 (Werkman STL8)

Offende Hyperman pSLc-HIF67-8

This is EXHIBIT FIERS-21
to
the Affidavit of Walter C. Fiers
sworn before me
this 13 th day of November, 2001

Commissioner for Oath or Notary Public

1

af Zaterdag)

Lakkoopcommando met NF4 pSRKBC7-12 D19
pSTL26-8
NF4 pBRKB-C7 M5219 pSRKBC7-12D19
M5219 pIRKB-C7 pSTL26-8

Voorgang: LB
Stuft van Hot medium (1/5 gefiltreerd)
blijft 7L op 20°C
dan stuft naar 42

Lakkoopcommando

~~42° [illegible]~~

- SRKBC7-12 / SRKBC7-12D19 } zend NF4 als M5219

42° : a) om 30-40 min
b) om 90-100 min
28° : om 90-100 min

- STL26-8 en NF4 en M5219

42° : a) om 90-100 min
b) om 120-190 min
28° : om 180-190 min

Label:

per typhoonmanta: 5 ml met 50 µCi ^{35}S-Met

cellen afcentrifugeren en toegevoegen in
100 µl | 80 mM Tris pH 7,4
10% glycerol
totaal volume gebracht op 1 150 µl

Verdere verwerking

A) Rechtstreeks op gel

15 µl cellen + 15 µl 2 x laemli kokend
laden op 12,5% acrylamide

gedaan van alle opvolgingen JTL 24-2
D19
SRKB 17-12

B) Immunoprecipitatie met volledige cellen

15 µl cellen + 15 µl | 8 mM Tris
10% glycerol
2% SDS
} 5 min koken

+ 400 µl H2O
+ 100 µl NETS + BSA

Verwerkt met Packard
Finale pellet 3x met 1x ETS gewassen
Immunoprecipitaat gekookt door kolom met
1x Laemli in evenredigheid van Stap A.

→ Uitgevoerd voor S7L29-8
A19

C) Combrische Blockatie

45 µl cellen centrifugeren en kwijt in
40 µl 2% Laure-EDTA

Klassieke Shock procedure

Voorbehandeling van 0S (na 40 µl)

- 13 µl gepreciptieerd met 1 µg Gitochroomdrager
en kaarten op 12,5% polyamide

- 10 µl 0S van immunoprecipitatie
of Patrick

D) Opzuiveringstechnieken

Bedoeling is de fosfotoxatie van gemerkte kinasen na te gaan bij verschillende opzuiveringsmethoden.

Med uitgevoerd op eindmonsters 42° en D19 ST129-8

8 µl van gelabeld materiaal werd gemengd met 1 ml OV kultuur SRKB (niet gelabeld).
- gepelletteerd in Eppendorf en ingevroren bij -80°C

Methoden

1°) Opzuivering met Lysozyme

Pellet in 110 µl HEPES buffer / 1 mg/ml Lyso 3 mM β-ME

- 30 min op 4°
- 1 x ontdooien CO_2 - methanol
- ontdooien 37°C
- 30 min 20.000 rpm SS34 adaptor

Supernatans: - 20 µl afgenomen + 20 µl 2x kleurb. → SDS

- rest voor immunoprecipitatie

[redacted]

9

2°) Opening met lysozym gedaan door 0,1% SDS

- lyse zoals onder 1°)
- één cleaning + DNS tot 0,1%
  (lyse licht troebel te zien)
- cleaning op 2u 4 30min (gaat niet zo goed; veel DNA bij.
- verder zoals A.

3°) Opening door sonicatie in eutank buffer

Pellet in 1 ml pH HEPES + 3 mM β-ME

Sonicatie op jo met dikke tip in PBS kuipje 11' met 15 onderbroken; (X

Verder zoals 1°)

4°) Opening door sonicatie in eigen buffer

Pellet in 100 µl pH 2, buffer
(cf. Rik Wierenga)

Zoals 3°) en verder 1°)

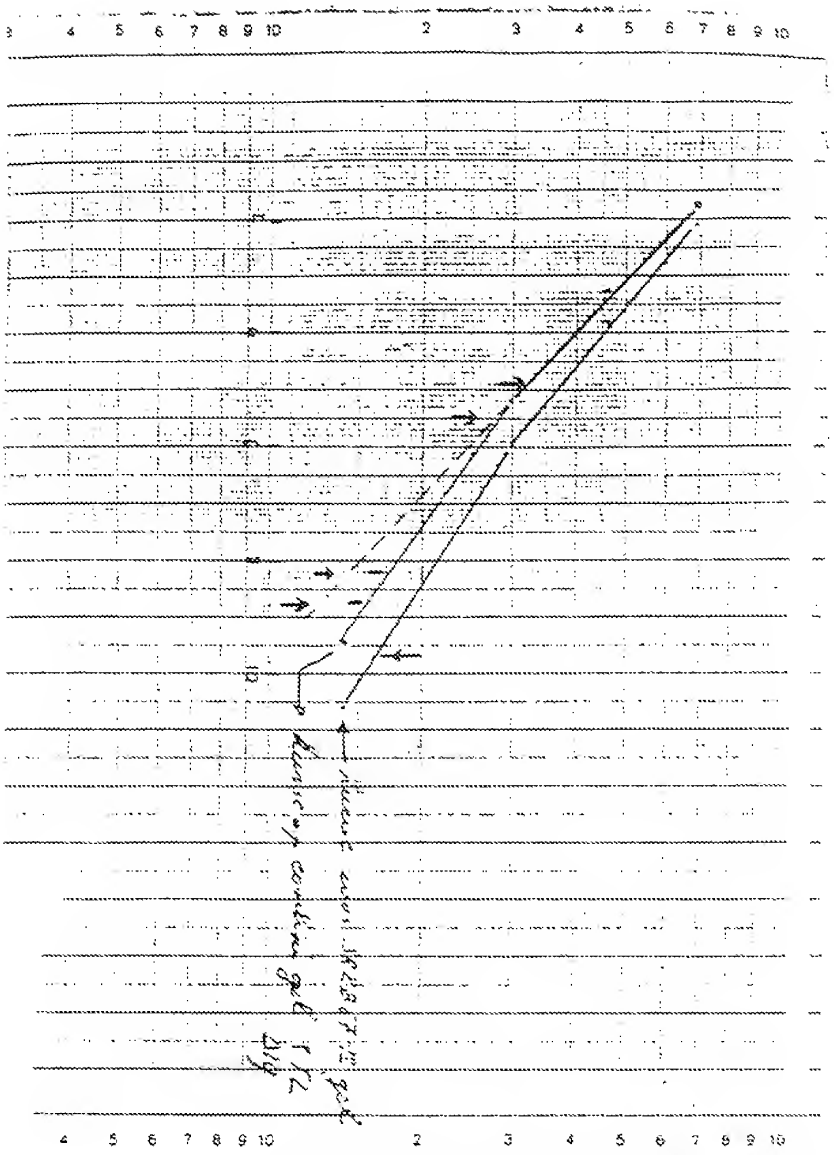
Lysing gecentrifugeerd met NaOH tot 0,02 M

Bespreking —

Autoradiogame van intakte cellen

Voor opmerkingen die vanuit de autoradiogram van kinetiek Experiment

Sequoia case M.5219 67-12



Eine Achse logarithmisch geteilt von 1 bis 1000, Einheit 90 mm, die andere in mm

Voorspelde lengte van fusieproducten

Δ19:

23 a.a.	β-Lactamase leader	
59 a.a.	mature β-Lact tot aan HincII	
1 a.a.	aan HincII 1F	
21 a.a.	leader 1F	
166 a.a.	mature 1F	
Totale Precursor:	270 a.a.	29110
- β-Lact-Leader	247 a.a.	26676
- Mature 1F	166 a.a.	17928
7L2	8	
9 a.a.	aan Replikase	
26 a.a.	conf. Bgl II — tot AUG	
21 a.a.	leader 1F	
166 a.a.	mature 1F	
Totale Precursor.	312 a.a.	33695

Dec. 30. '97
C. Fiers
M. Gysler

HSL

TGG, GAT, GTT, CAG, TTT, CGG, AGG, CAA, CCT, TTC,

GAA, GCC, TTT, GCT, CTG, GCA, CAA, CAG, GTA, ~~GTA~~, GTA

~~GAC~~, GAC, ACT, GTT, CGT, GTT, GTC, AAC AUG

~~GGC~~,

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sworn before me
this 13 th day of November, 2001

Commissioner for Oath or Notary Public

Kinetiekexperiment

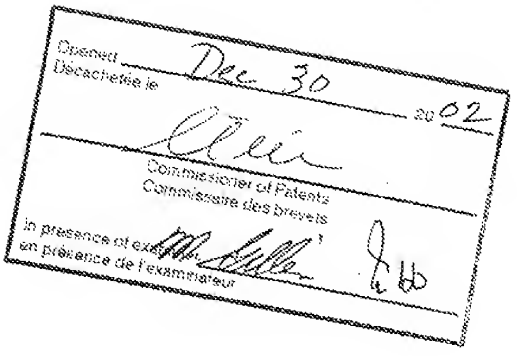
H5219 p PLC HFIF 17-12D19 (D19)
p PLC HFIF 17-8 (STL8)

Cellen in LB 28°C tot 2.10^8
Toevoegen aan Met⁻ medium (~~glucose~~).
Na 60 min schudden t=0

Labeltijden: 28° 30' } 1 ml met 5 µCi ^{35}S
42° 30'
60' } gelabeld 10 min.
120'
150'
180'
27' 10'

Cellen gehalveerd door centrifugatie en opgeschort in 10 µl Laemli buffer

Gel: 10 µl geladen



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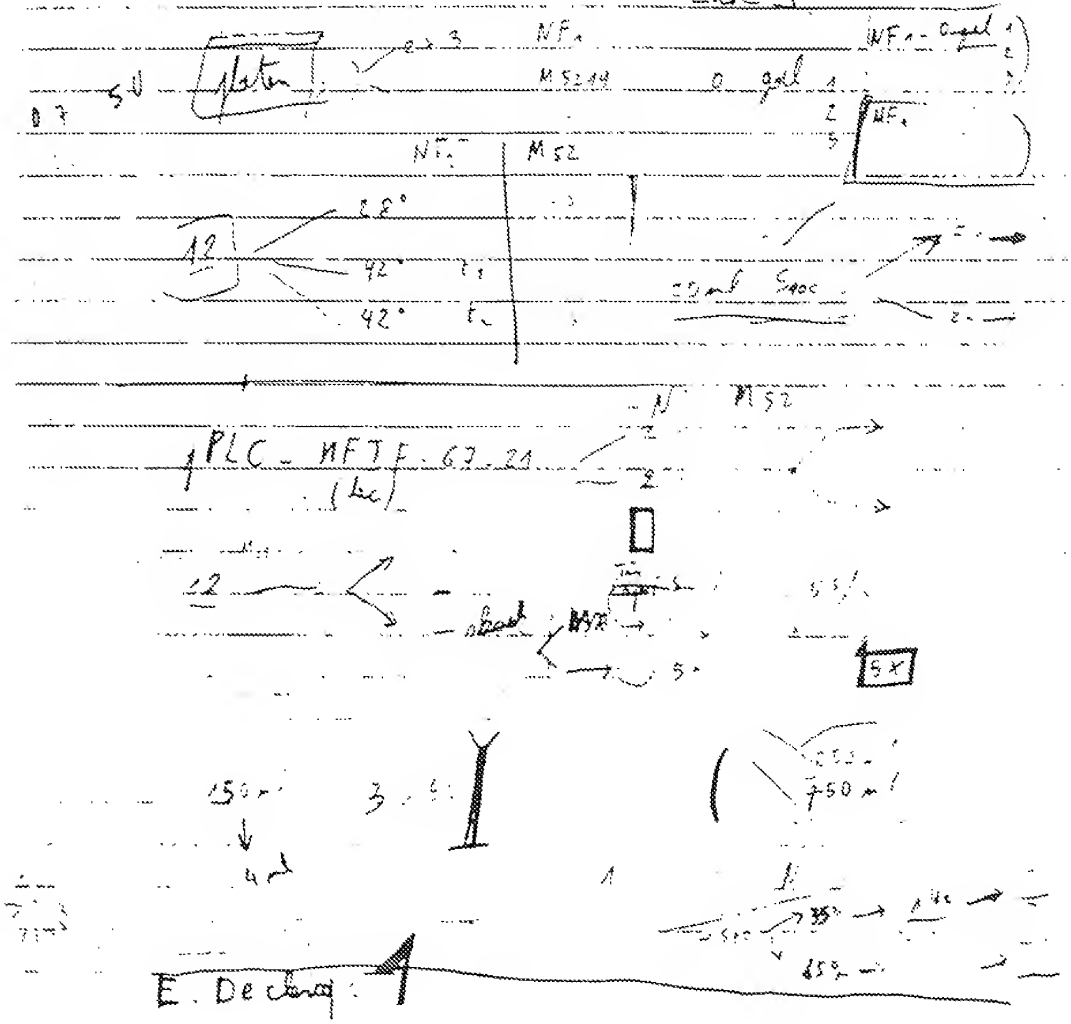
26/4/80

Inductie

Na bestuderen van de resultaten van de tests uit Leuven worden volgende inducties gepland:

- y/p PLa - HFIF - 67 - 12 zowel in MS219 als in NF2
- y/p PLC - HFIF - 67 - 11 met bac-operator in het rek L



E. Declercq

4k

Verslag van Biologische resultaten

Er zit geen overeenkomst tussen die van Leuven en Brussel. Nochtans zijn het geen "onwenselijke" resultaten als vorige week.

Het voornaamste is dat er, zowel met in overeenkomst met Brussel, ~~geen~~ een gelijke biologische activiteit ~~was~~ is in extracten van pRKa-HFIC-67-12 / pRKa B"-67-22 / lege kandidaat van de recombinatie / een titer (0.2) / activiteit in overeenkomen in de HFE2 (na inductie), net zoveel (0.3) in de HFE19 (na inductie). De 25°C kweken geven <0.2. Het voornaamste is echter dat de activiteit in E.SM <1.0 is. Dat is zelfs niet voornaamste. De herhaling van het exp. met cytokinemeting volgt volgende week.

P.S. Normaal diende volgende week geen enquête in Leuven (zowel) Brussel van recht – het kwam toch op vrijdag (tot vrijdagavond) 17 februari 1989 zijn dat de twee klassen kombakken om toch de enquête te kunnen laten vullen, Bedankt.

Rik

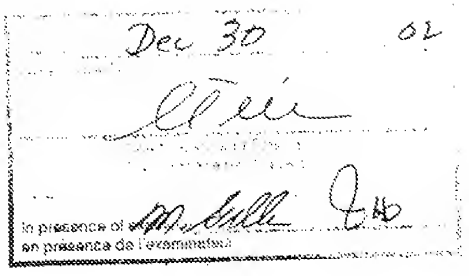
(Ik kom vandaag de proeven doen met handschoen)

Konstruktie met lac Operator.

① Analyse van drie positieve kolonies
Slechts 1 bruikbare kandidaat. Hiervan wordt de oriëntatie maandag bepaald.
Inductie gepland voor maandag.

② Hernemen konstruktie met lac-Operator, na recovery van EcoRI fragment.
Konstruktie gemaakt in pPLa HFIF 67-1 wegens gebrek aan DNA van pPLc HFIF-67-11.
Transformatie gebeurt maandag in K12.

E. Seiman.



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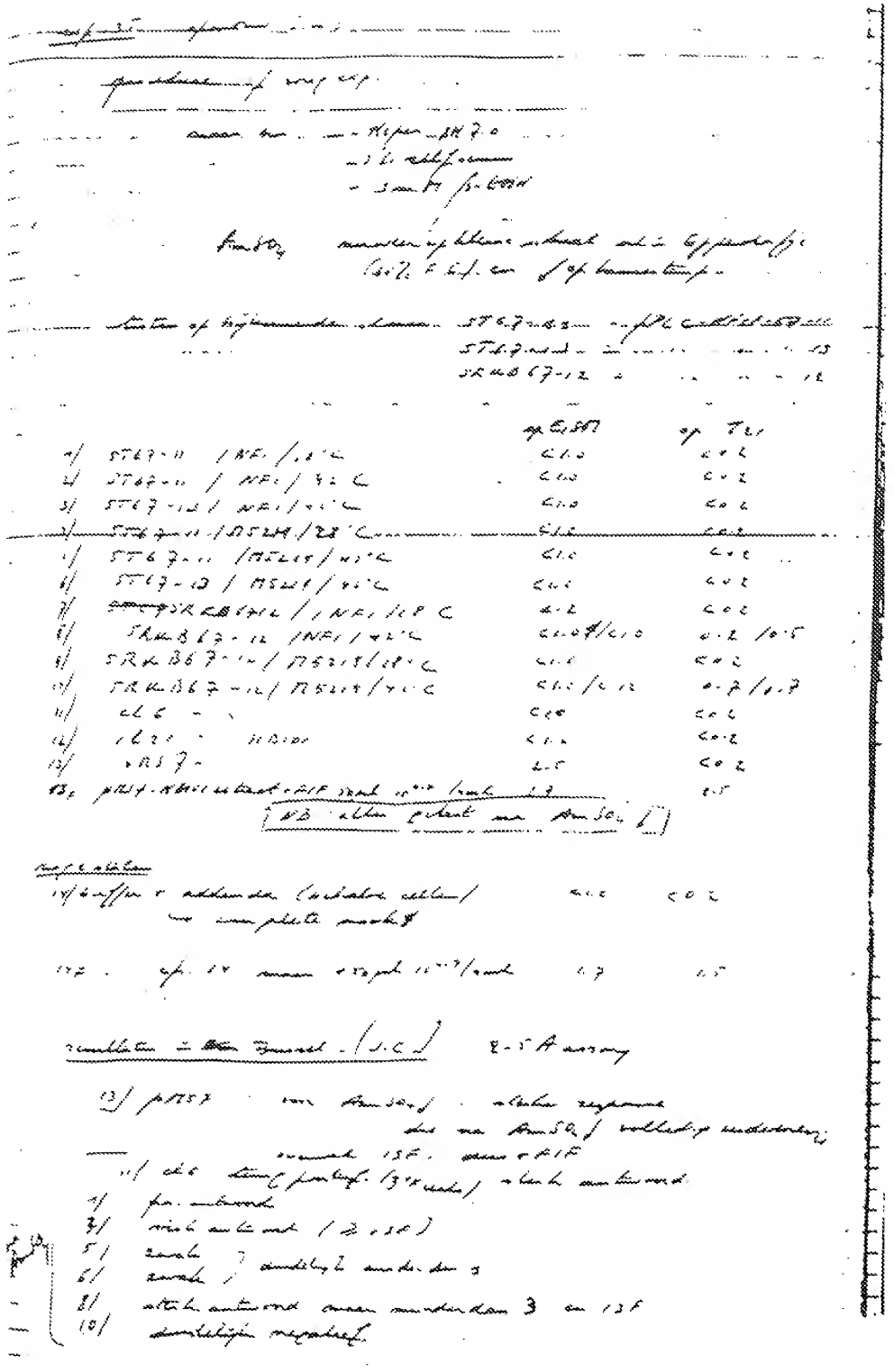
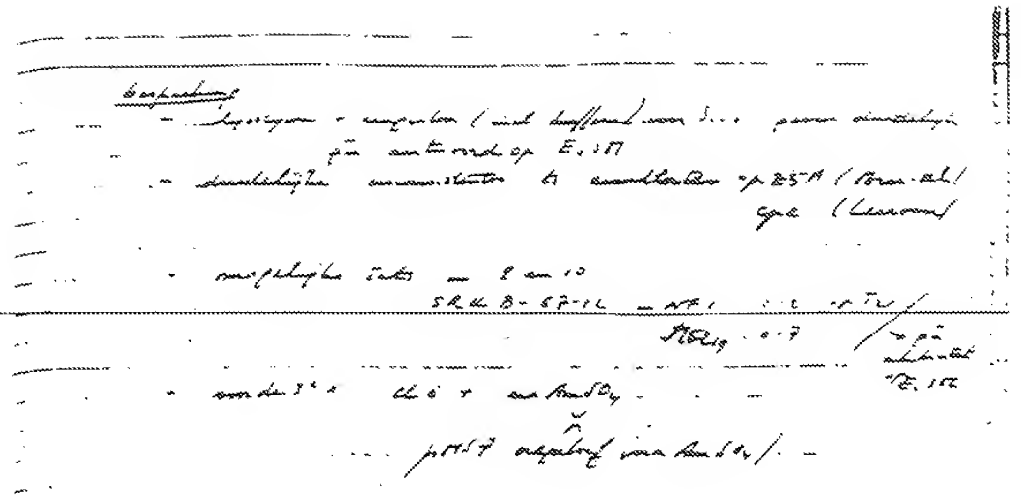




3/ [illegible] ... PBS + 0.1% SDS ...
5 ml ... pH 7.3 ... + 2% gelatin
fractions ~15 drops per 2 pyrolled

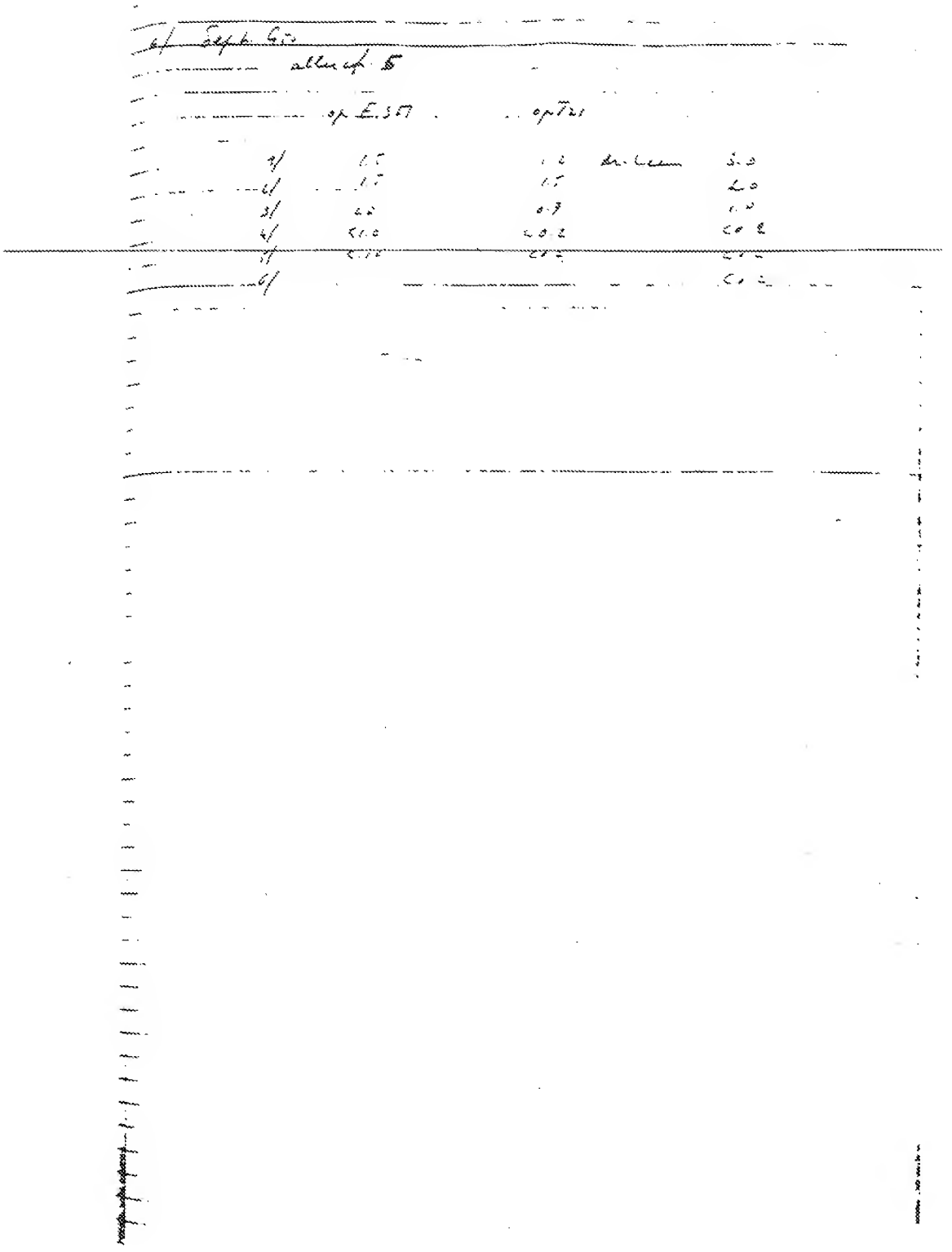
	E, SM	Tu		NB SDS [illegible]
[illegible] 1/	<1.0	<0.2 dr blank	3.0	[illegible]
2/	<1.2	<0.2	4.0	[illegible]
3/	2.5	2.7	[illegible]	
4/	2.6	2.7	3.0	
5/	2.2	2.0	2.5	
6/			[illegible]	
8/			[illegible]	
9/			[illegible]	
10/			[illegible]	
11/			2.5	
12/			2.5	

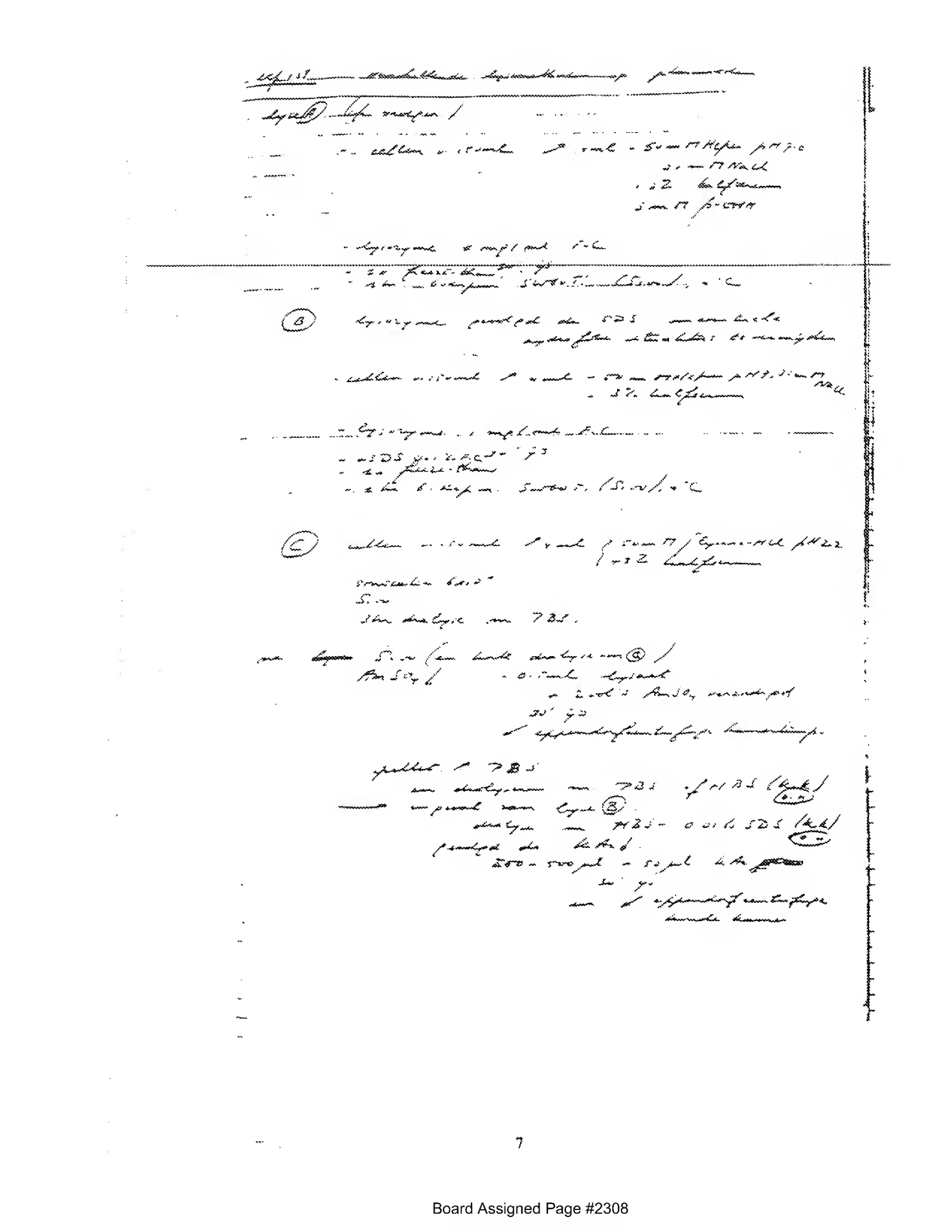
4 Sephadex G50 Ø 7mm, h = 20cm in PBS + 0.1% SDS, 6.0
5 ml ... pH 7 + 2% gelatin
fractions ~15 drops per 2 pyrolled
NB. SDS [illegible]

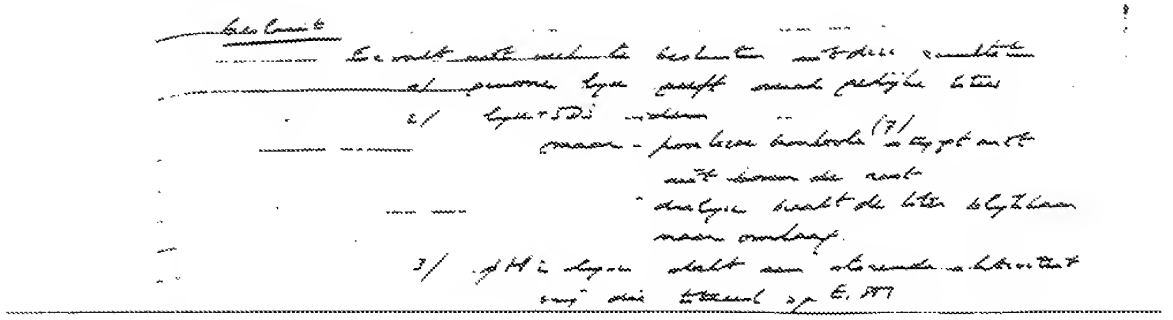
	op E, SM	op Tu		
[illegible] 1/	<1.0	<0.2	dr blank	[illegible]
2/	<1.2	<0.2		[illegible]
3/	<1.0	<0.2		[illegible]
4/	<1.0	<0.2		[illegible]
5/	<1.0	<0.2		[illegible]
6/		[struck out]		[illegible]

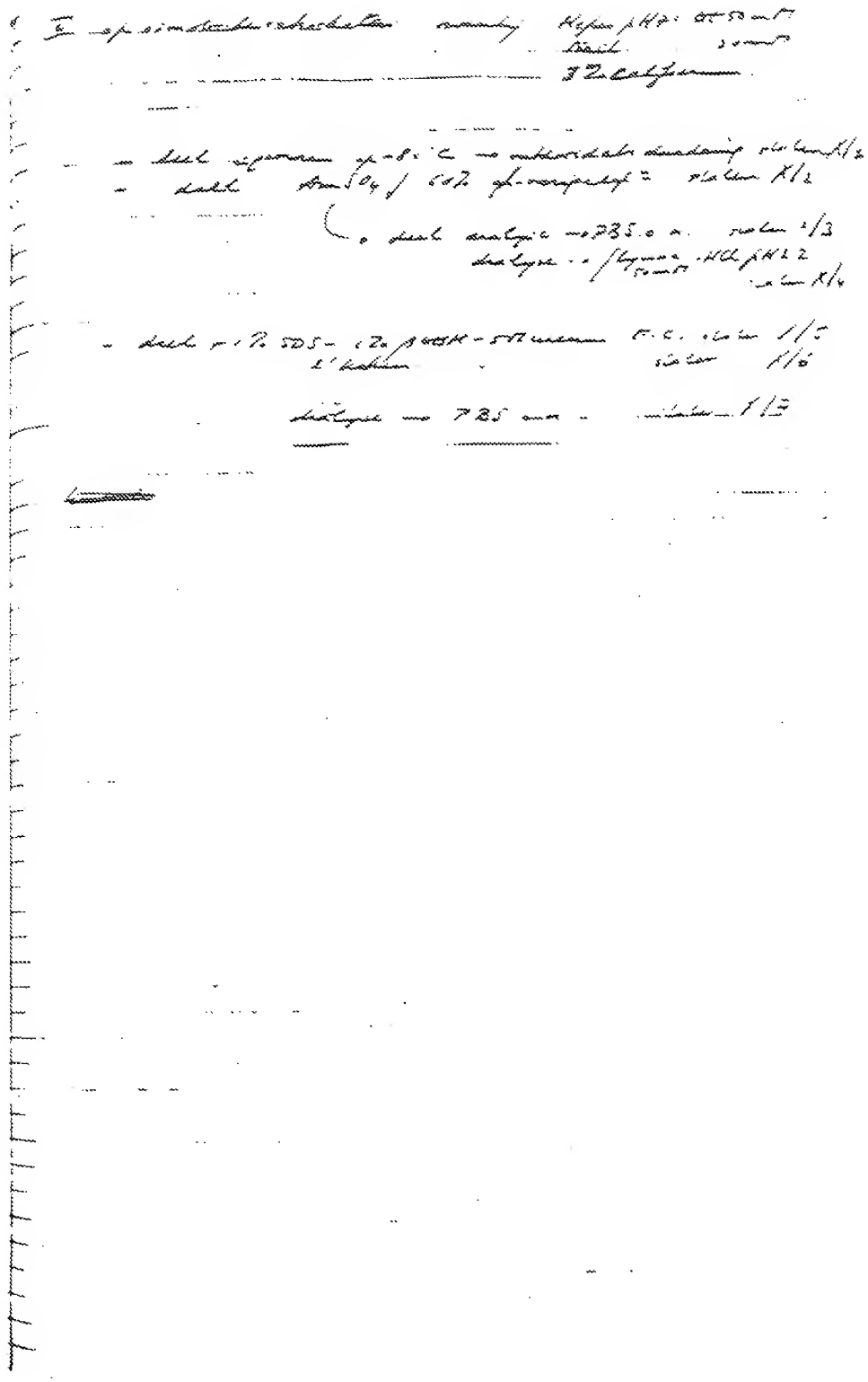
5/ AcA 54 Ø 7mm h = 20cm ... 0.05M glycine-HCl pH 2.2.
5 ml + pH 7 + 2% ... pH 2.2, [struck out] [illegible]
fractions ~15 drops per 2 pyrolled

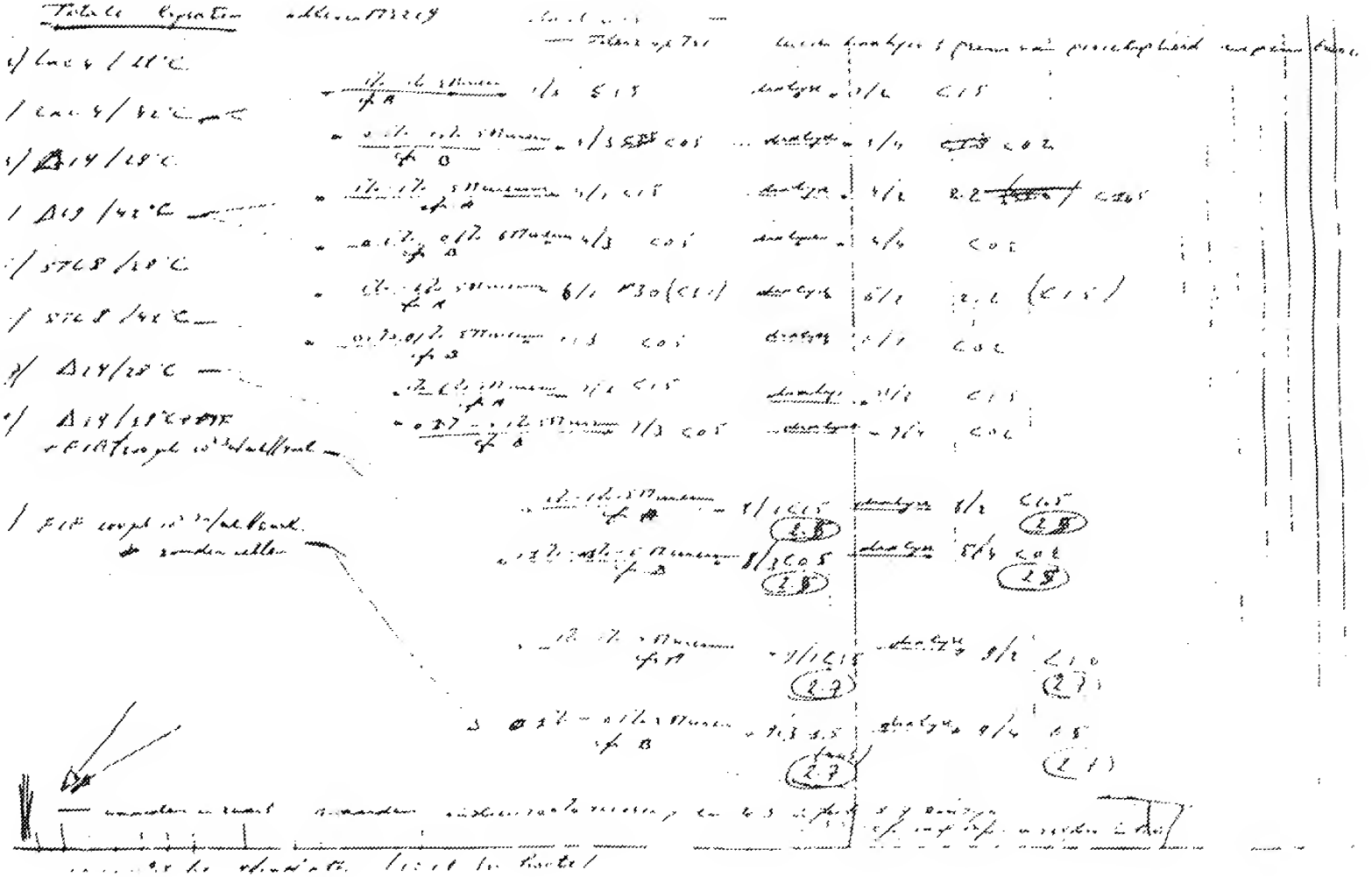
	op E, SM	op Tu		
1/	<1.0	<0.2	dr blank	[illegible]
2/	<1.0	<0.2		[illegible]
3/	<1.0	<0.2		[illegible]
[illegible] 4/	1.0	<0.2		[illegible]
5/	1.7	1.7		[illegible]
6/	2.0	1.5		[illegible]
7/				[illegible]
8/				[illegible]
9/				[illegible]
10/				[illegible]
11/				[illegible]
12/				[illegible]











[illegible] exp't [illegible] [illegible] ([illegible] 5.1 [illegible])

[illegible]

1/ [illegible] [illegible] [illegible]

2/ < 2

2/ [illegible] [illegible] ≈ [illegible] [illegible] 6/2 (≈ 3.0)

3/ < 5 < 2 (≈ 0.55)

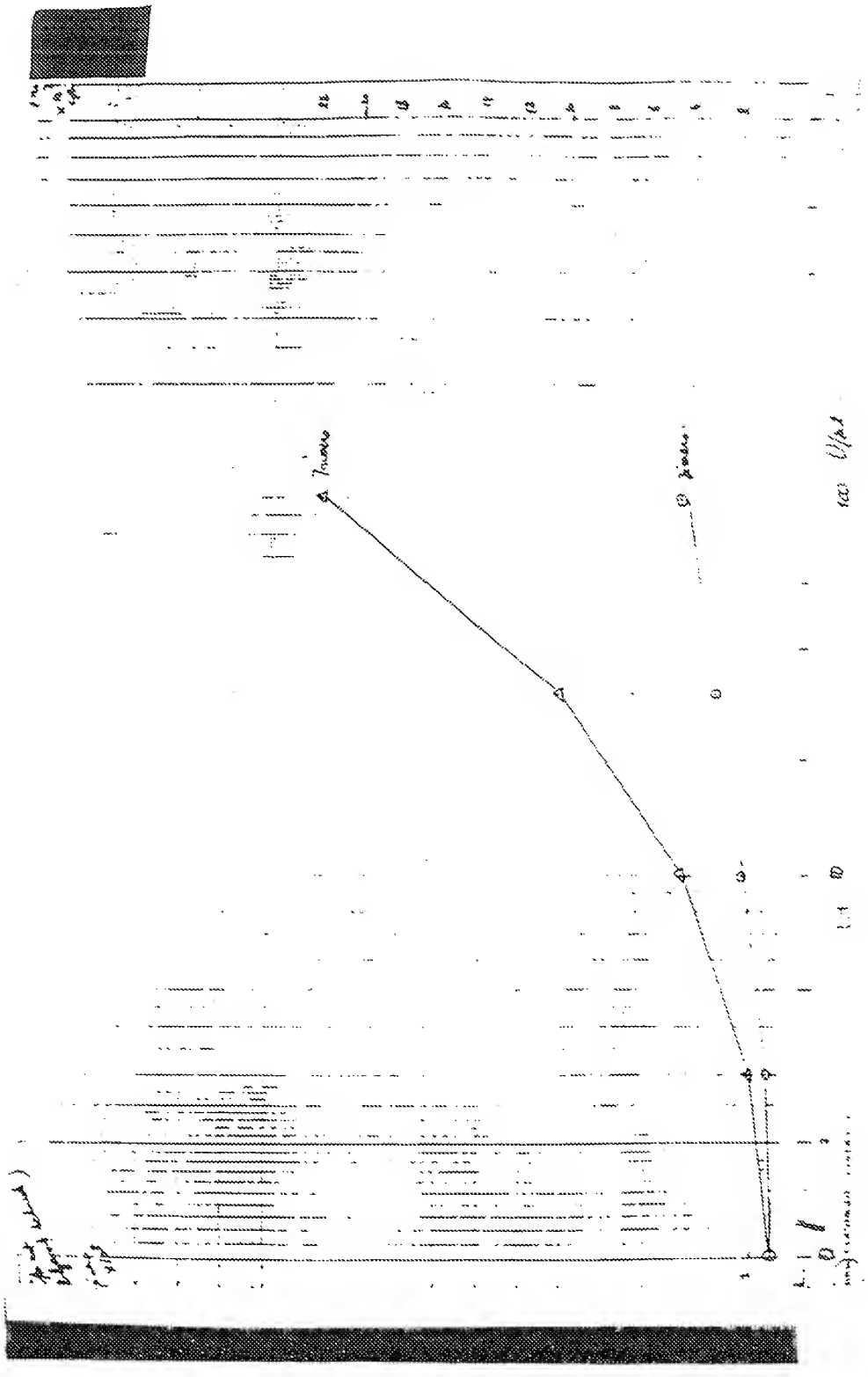
4/ [illegible] [illegible] [illegible] ≈ [illegible] [illegible] 15/2 (≈ 2.5)

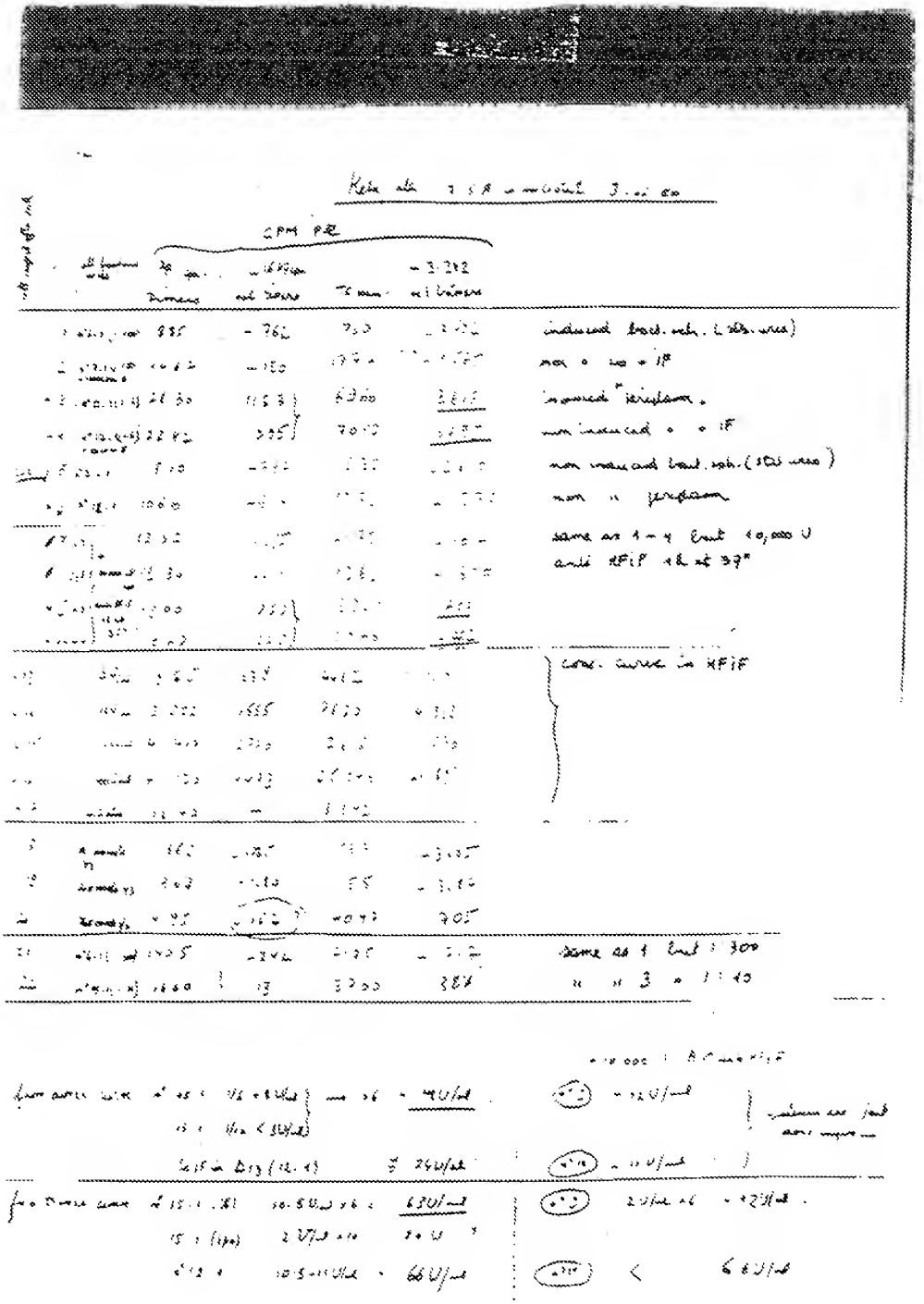
	[illegible]	Fe	BSC	PRA	[illegible]	Tu	ESR	[illegible]
[illegible]	< 2.0	< 2	< 2	≤ 2	< 2	3.5	2.5	[illegible]
[illegible]	< 2.0	< 2	< 2	< 2	< 2	3.3	2.5	[illegible]
3/ [illegible]	< 1.0	< 1	< 1	≤ 2	< 2	2.5	[illegible]	[illegible]
4/ [illegible]	< 1.0	< 2	< 1	< 2	< 2	[illegible]	2.8	2.0
[illegible]	3.0	2.8	~~[illegible]~~ 2.5	[illegible]	1.5	3.8	2.3	[illegible]
[illegible]	2.3	2.5	~~[illegible]~~ 2.2	[illegible]	2.5	3.5	1.8	3.0
[illegible]	< 0.2	2.2	0.1	~~[illegible]~~ 0	0	3.5	3.3	2.5

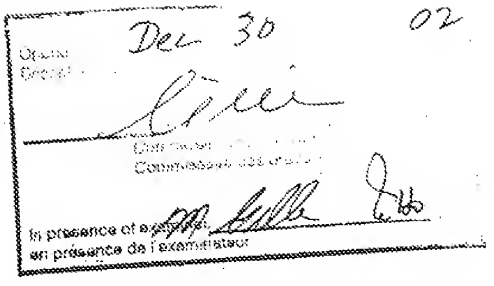
i/ [illegible]

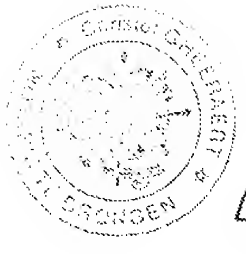
6/2 [illegible] $10^{-5.2}$

15/2 [illegible] $10^{-5.3}$









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to
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this 19 th day of November, 2001

Commissioner for Oath or Notary Public

Proc. Natl. Acad. Sci. USA
Vol. 77, No. 6, pt. 1, 3230–3233, September 1980
Biochemistry

Expression of the human fibroblast interferon gene in *Escherichia coli**

(hybrid ribosome-binding site/antiviral activity/*lacZ* gene fusions/portable promoter)

TADATSUGU TANIGUCHI† AND LEONARD GUARENTE‡, THOMAS M. ROBERTS‡, DAVID KIMELMAN‡, JOHN DOUHAN III‡, AND MARK PTASHNE‡

†Cancer Institute, Japanese Foundation for Cancer Research, Toshima-Ku, Tokyo 170, Japan; and ‡The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Contributed by Mark Ptashne, June 25, 1980

ABSTRACT We applied the method of Guarente *et al.* [Guarente, L., Lauer, G., Roberts, T. M. & Ptashne, M. (1980) *Cell* 20, 543–553] to construct plasmids that direct expression in *Escherichia coli* of the human fibroblast interferon (F-IF) gene. Two plasmids were recovered. One directs efficient synthesis of a protein whose primary sequence is that of pre-F-IF and the other, that of mature F-IF. Extracts of bacteria synthesizing mature F-IF display antiviral activity characteristic of human F-IF. This activity is lower than that expected from the differential rate of synthesis of the protein. We have detected no such activity in extracts of bacteria synthesizing pre-F-IF.

Human fibroblast interferon (F-IF) is a glycoprotein produced by human fibroblasts in response to virus and certain polynucleotides (1, 2). The secreted protein has potent antiviral activity that is readily assayed *in vitro* (2, 3). The sequence of the amino-terminal 15 amino acids of F-IF has been reported (4).

A cDNA molecule encoding human F-IF was cloned by Taniguchi *et al.* (5). The DNA sequence of this molecule predicts that the secreted F-IF contains 166 amino acids, the first 13 of which would be identical to the corresponding sequence of the protein as determined by Knight *et al.* (4). Moreover, the sequence is consistent with the idea that F-IF is synthesized as a precursor (pre-F-IF) with a 21-amino-acid hydrophobic leader at its amino terminus (6, 7).

A series of papers from this laboratory* has developed methods to express cloned prokaryotic and eukaryotic genes in *Escherichia coli* (8–11). The protein products of these plasmid-carried genes were produced in their native states—that is, unfused to other proteins (8–11). The essential feature of this method is to position a "portable promoter" in front of the cloned gene so that the gene is efficiently transcribed and the resultant mRNA is efficiently translated, beginning at an initiation codon—e.g., AUG. This AUG may, but need not be, that which directs initiation of synthesis of the native protein *in vivo*. The procedure of Roberts *et al.* (9, 10) enables us to position the portable promoter at various positions in front of the cloned gene by using recombination *in vitro*. The method of Guarente *et al.* (11) exploits *lac* genetics to identify those positionings that direct efficient transcription and translation of the cloned gene. This latter procedure eliminates the need for any assay for the gene product to identify those bacteria that express the desired proteins (see *Method of Gene Expression* in *Results*).

The protein and DNA sequence data referred to above indicate that both F-IF and pre-F-IF bear methionine residues at their amino termini (4, 6). We describe in this paper the application of the method of Guarente *et al.* (11) to the F-IF gene. We describe the construction and identification of plasmids that direct the efficient synthesis of two proteins. The primary sequences apparently correspond to the sequence of F-IF in one case and to that of pre-F-IF in the other. F-IF produced in bacteria prevents viral growth as assayed *in vitro*.

MATERIALS AND METHODS

DNA Constructions. All techniques were as described by Guarente *et al.* (11). pTR56 (see Fig. 1) was constructed in two steps as follows. First, a plasmid (pLG111) was constructed that bears a *Hin*dIII synthetic linker three nucleotides before the ATG of pre-F-IF (6). This was accomplished by joining four DNA fragments: (i) a *Bam*HI–*Pst* I backbone fragment from pLG300 (11); (ii) a *Hin*dII–*Bgl* II fragment containing the entire F-IF coding sequence from TpIF319-13 (5); (iii) a *Pst* I–*Pvu* II fragment from pGL101 bearing the 5′ end of the *amp* gene; and (iv) a *Hin*dIII linker. Ligation of these fragments fuses two complementary sticky ends (*Pst* I–*Pst* I and *Bam*HI–*Bgl* II) and two blunt ends (*Pvu* II–*Hin*dIII linker and *Hin*dIII linker–*Hin*dII). The *Pst* I joining thus reconstitutes *amp*. Second, pTR56 was constructed by joining three fragments: (i) a *Pst* I–*Pst* I fragment from pLG111 bearing the 5′ portion of *amp* and the 5′ portion of the F-IF gene; (ii) an internal fragment of the F-IF gene extending from the *Pst* I site to the first downstream *Hin*f site (6), the latter having been rendered flush by DNA polymerase I (12); and (iii) a *Pst* I–*Bam*HI fragment from pLG300 (11) that bears the 3′ end of *amp* and a 3′ fragment of *lacZ*. The *Bam*HI end of this fragment had been rendered flush by DNA polymerase I (12). Ligation of these fragments generates two *Pst* I–*Pst* I fusions, one of which reconstitutes *amp*, as well as a *Bam*HI (filled in)–*Hin*dIII (filled in) fusion joining the 5′ portion of the F-IF gene in phase with the 3′ portion of *lacZ* (cf. refs. 6 and 11).

Radiolabeling of Proteins. The procedure has been described (11). Pulse labeling was with 300 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine and chasing was achieved by adding a 1000-fold excess of unlabeled methionine. This was done at 30°C. Labeled extracts were run on 15% acrylamide gels for analysis as described (13).

Preparation of Bacterial Extracts. Extracts were prepared essentially as described by Nagata *et al.* (14) except phenylmethylsulfonyl fluoride and EDTA were added in the lysis

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Abbreviations: F-IF, fibroblast interferon; SD, Shine–Dalgarno

* The work described herein was carried out by the authors at The Biological Laboratories, Harvard University.

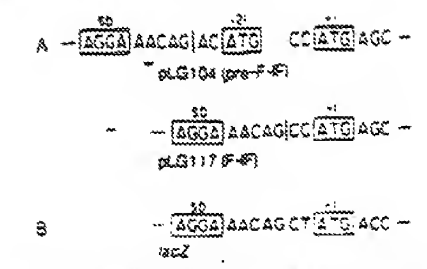

FIG. 3. (A) Nucleotide sequences of the DNAs around the regions encoding ribosome-binding sites of genes encoding pre-F-IF (in pLG104R) and mature F-IF (in pLG117R). The vertical lines separate sequences carried on the *lac* portable fragment from F-IF sequences. The boxes indicate the *lacZ* SD sequence and the ATGs encoding the amino-terminal methionines of pre-F-IF and of mature F-IF. (B) Corresponding region of wild-type *lacZ* (17).

(Fig. 2C, see also Fig. 3). The DNA containing the *lacZ* 5′ gene fragment was removed and replaced with the 3′ end of the F-IF gene, regenerating intact F-IF (pLG117R) and pre-F-IF (pLG104R), in which R indicates the reconstituted F-IF gene (Fig. 2D).

Plasmids that Direct the Synthesis of F-IF (pLG117R) and pre-F-IF (pLG104R). Fig. 3A shows the DNA sequence around the junctions of the portable promoter and the ATGs encoding the amino terminus of pre-F-IF (pLG104 and pLG104R) and that of mature F-IF (pLG117 and pLG117R). In each case, the SD sequence (AGGA) of *lacZ* carried on the portable promoter has been positioned seven base pairs from the ATG of F-IF. This is precisely the distance between the SD sequence and the ATG found in wild-type *lacZ* (Fig. 3B) (17). These particular placements were rare. In the screening that yielded pLG104, lactose-utilizing colonies appeared at a frequency of approximately 3%. Plasmid pLG117 was identified in a separate experiment involving more extensive exonucleolytic digestion. In this case, lactose-utilizing colonies were found at a frequency of only approximately 0.01%.†

Proteins Produced by pLG104R and pLG117R. The experiment of Fig. 4 uses the "maxicell" technique to display those proteins encoded by pLG104R and pLG117R. Suitably treated maxicells differentially incorporate radioactive amino acids into plasmid-encoded proteins that are easily visualized by autoradiography after polyacrylamide gel electrophoresis (11, 18). pLG104R and pLG117R each direct the synthesis of one protein in addition to β-lactamase. In the case of pLG117R, a protein was produced with a molecular weight of approximately 20,000, consistent with the predicted (from the DNA sequence) size of unglycosylated mature F-IF (6). In the case of pLG104R, the protein produced had a molecular weight of about 23,000, which corresponds to the predicted molecular weight of unglycosylated pre-F-IF.

Fig. 4 also shows the fate of pulse-labeled pLG104R and pLG117R proteins in a maxicell experiment. Densitometry tracing of the gel (not shown) reveals that pre-F-IF was completely degraded in a 50-min chase (lanes 3 and 3), and F-IF was about 50% degraded in that time (lanes 4 and 6). Although processing of β-lactamase from pre-β-lactamase is evident, pre-F-IF is apparently not processed.

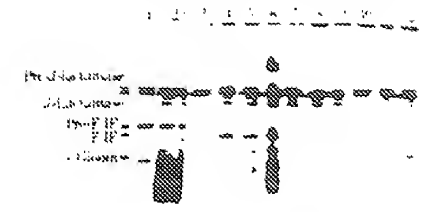

FIG. 4. Proteins encoded by plasmids pLG104R (pre-F-IF) and PLG117R (F-IF). Plasmid proteins were labeled by the maxicell technique (18). Proteins encoded by pLG104R were labeled for 5 min with [^{35}S]methionine (lane 1) and then chased with nonradioactive methionine for 12 min (lane 2) or 50 min (lane 3). Labeling and chasing were terminated by freezing the cells. After spinning for 2 min in an Eppendorf centrifuge, the cell pellets were suspended in Laemmli sample buffer (13), incubated for 5 min at 100°C, then subjected to analysis by polyacrylamide gel electrophoresis as described (13). Similarly, proteins encoded by pLG117R were labeled for 5 min (lane 4) and chased for 12 min (lane 5) or 50 min (lane 6). Lanes 7, 8, and 9 display the same labeling protocol performed on plasmid pCL101, which encodes only β-lactamase. Plasmid pLG302-2R, which directs synthesis of rabbit β-globin (5000–7500 molecules per cell) (11), was likewise analyzed as shown in lanes 10, 11, and 12.

We estimated the level of our F-IF and pre-F-IF synthesis in two ways. First, we measured the amount of radioactivity incorporated during a 5-min pulse into F-IF and pre-F-IF in a maxicell experiment. We compared these values with a known standard, namely, rabbit β-globin synthesized by the plasmid pLG302-2R (11) (see Fig. 4). This comparison suggests that, were the F-IF molecules stable, the steady-state levels would be 5000–10,000 molecules per cell. Second, we found that growing cells bearing plasmids pLG117 and pLG104 synthesize about 1200–1400 units of β-galactosidase (19). Assuming that the hybrid F-IF (or pre-F-IF)–β-galactosidase molecules have the same specific activity as β-galactosidase, this value represents 5000–10,000 molecules per cell (11). Previous experience with β-galactosidase hybrid proteins modified at their amino termini suggests that they are stable during growth (20).

Antiviral Activity of the Human Fibroblast Interferon Polypeptide Synthesized in *E. coli*. Extracts of bacteria bearing plasmid pLG117R inhibited growth of vesicular stomatitus virus on human fibroblast cells in a typical interferon assay (inhibition of cytopathic effect) (2, 21). This activity was abolished by antibody to F-IF but not by antibody to leukocyte interferon (Fig. 5). Moreover, extracts of bacteria carrying pLG104R, pLG115R, and pBR322 failed to manifest antiviral activity. The antiviral activity directed by pLG117R survives a sojourn at low pH (pH 2.0) or treatment with DNase and RNase, but it is abolished by trypsin treatment (data not shown). Assuming that unglycosylated F-IF is as active as glycosylated F-IF (2×10^8 units/mg (22)), the activity we typically recovered would correspond to approximately 50 interferon molecules per bacterial cell.

DISCUSSION

Our results strongly suggest that our application of the method of Guarente *et al.* (11) to the F-IF gene isolated by Taniguchi *et al.* (6, 7) has produced two plasmids (pLG104R and pLG117R) that direct the synthesis of proteins whose primary sequences correspond, respectively, to those of pre-F-IF and F-IF.

† This screening also yielded a third fusion, pLG115. In this case, the portable promoter is abutted to nucleotide 18 of the sequence encoding the F-IF leader some 20 bases from the nearest possible initiator triplet (6). Experiments similar to the experiment of Fig. 4 suggest that, in this case, protein synthesis initiates at the internal ATG located at position 175 in the DNA sequence published by Taniguchi *et al.* (6, 7). This ATG is fortuitously preceded by an SD-like sequence in the F-IF gene. We do not understand how the portable promoter placement in pLG115 enhances the efficiency of utilization of this ATG.

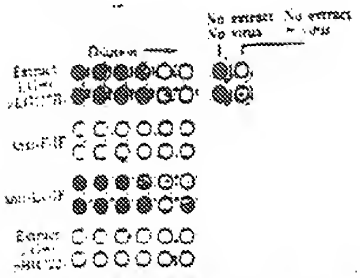

FIG. 5. Characterization of antiviral action of bacterially produced F-IF as assayed *in vitro*. Extracts of bacteria bearing pLG117R were added in 1/2 dilutions to human fibroblast cells (FS-7) growing in microtiter dishes. In two cases, these extracts were pretreated separately with antibody to F-IF and with antibody to leukocyte interferon (Le-IF). The treated cells were challenged with vesicular stomatitus virus and stained with crystal violet (2). Wells containing cells uninfected with virus or protected against viral infection stain darkly with this dye. Also shown are the effects of no extract and an extract of a strain bearing plasmid pBR322.

These plasmids bear a *lac* portable promoter abutted to the ATG encoding the amino-terminal methionine of pre-F-IF (pLG104R) and to the ATG encoding the amino-terminal methionine of F-IF (pLG117R). In each case, these promoter placements were originally recognized by their abilities to efficiently direct synthesis of a F-IF-β-galactosidase hybrid protein. In each case, the distance separating the SD sequence of the *lac* promoter from the ATG is precisely that found in the case of wild-type *lacZ*. When compared with pBR322, pLG117R and pLG104R each direct synthesis of one new protein of molecular weights approximately 20,000 and 23,000, respectively. These are the sizes expected for unglycosylated proteins with the primary sequences of F-IF and pre-F-IF as predicted from the DNA sequence of Taniguchi *et al.* (6). In previous cases, we have found that formation of such hybrid ribosome-binding sites, not dissimilar to the ones shown here, have directed correct initiation of protein synthesis (rabbit β-globin, simian virus 40 tumor (t) antigen, λ repressor) as determined by direct amino acid sequencing (8–11). In all of these cases the amino-terminal methionine was maintained. We have not determined the amino acid sequence of our bacterially produced F-IF.

Plasmid pLG117R, but no other plasmid described here, directs antiviral activity characteristic of F-IF under our assay condition. The amount of this activity is much lower (only about 1%) than that predicted on the basis of the rate at which the protein is synthesized in our bacteria. We imagine the following possible explanations for this difference.

(i) The protein is rapidly degraded. The pulse–chase experiment of Fig. 5 indicates that the bacterially produced F-IF protein is somewhat unstable under the particular conditions of that experiment. But this degree of instability would not account for the differences between the expected and the observed result. The conditions under which we visualized the proteins (i.e., in minicells) may not accurately reflect the extent of degradation in growing cells.

(ii) Bacterially synthesized F-IF, which is unglycosylated, may have low specific activity in our *in vitro* assay.

(iii) Our method of extraction may not efficiently recover active F-IF. We have not systematically varied growth conditions or methods of extraction.

We have only hints as to why pre-F-IF, synthesis of which is directed by pLG104R, is totally inactive in our assay. It is possible that the unprocessed form is inherently inactive. The minicell experiments show no indication that pre-F-IF is correctly processed and suggest that it is hypersensitive compared to F-IF. Oxender *et al.* (23) have described a case (the leucine-specific binding protein) in which the mature form is less sensitive to proteolytic degradation than is the precursor bearing a hydrophobic leader. It is possible that pre-F-IF is exported to the periplasm with or without concomitant cleavage of its leader and is rapidly destroyed there.

We are indebted to Dr. J. Vilcek for his help and supply of antibodies. The work of T.T. has been supported by a grant from the Cancer Institute, and T.T. thanks Drs. H. Sugano and M. Muramatsu for their encouragement. L.C. is a Postdoctoral Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

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Commissaire des brevets

In presence of examiner
en présence de l'examinateur

This is EXHIBIT FIERS-26
to
the Affidavit of Walter C. Fiers
sworn before me
this 9 th day of November, 2001

Commissioner for Oath or Notary Public

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ARTICLES

Expression of human fibroblast interferon gene in *Escherichia coli*

Rik Derynck*, Erik Remaut*, Erik Saman*, Patrick Stanssens*, Erik De Clercq† Jean Content§ & Walter Fiers*

* Laboratory of Molecular Biology, State University of Ghent, Ledeganckstraat 35, B-9000 Ghent, Belgium
† Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium
§ Institut Pasteur du Brabant, rue du Remorquem 28, B-1040 Brussels, Belgium

The human fibroblast interferon gene was inserted in a thermoinducible expression plasmid under control of the pha lambda P_L promotor. The primary translation products predicted on the basis of the plasmid constructions were hyb proteins starting with β-lactamase or phage MS2 polymerase information followed by the total preinterferon. On inducti antiviral activity, whose physico-chemical, immunological and biological characteristics closely corresponded to those authentic human fibroblast interferon, was synthesized. Processing to a size compatible with mature but unglycosyla authentic product was observed.

On exposure to viruses or other specific inducers most vertebrate cells secrete protein(s) with broad antiviral action known as interferons. Human interferons are being intensively studied for their antiviral[1], anticellular[2] and immunomodulating[3] activities. Clinical trials have been carried out mainly with leukocyte interferon but also with fibroblast interferon, and some promising results have been obtained with both types in the treatment of viral diseases and cancer[1,4-9]. Tests with fibroblast interferon have been severely restricted by its very limited availability.

In a previous report[10], we described the construction and characterization of chimaeric plasmids containing human fibroblast interferon (HF-IF) cDNA. Two other groups have constructed plasmids containing either human leukocyte[11] or human fibroblast[12] interferon cDNA and in the former case, interferon-related polypeptides, as judged by biological and immunological criteria, were detected in *E. coli* strains harbouring the chimaeric plasmids. We have now inserted the HF-IF coding sequence derived from our original clones into appropriate sites on specifically constructed expression vehicles which contain the strong leftward promoter (P_L) of bacteriophage λ. The functioning of the promoter could be controlled by using host strains which synthesize a temperature-sensitive repressor (cI-ts). We describe here how plasmids containing P_L in front of the HF-IF coding sequence direct the synthesis of polypeptides with human fibroblast interferon activity in *E. coli*.

Construction of plasmids allowing expression of HF-IF

Construction of the different plasmids containing HF-IF DNA under the control of lambda P_L is schematically represented in Fig. 1. The formation and use of acceptor plasmids pPLa2311, pPLa8 and pPLc24 will be published in detail elsewhere (E. Remaut *et al.*, in preparation).

None of the chimaeric plasmids previously described contains an uninterrupted HF-IF gene[10]. A complete and continuous coding sequence for HF-IF was reconstituted by inserting an *Eco*RI-*Pst*I fragment from pHFIF-6 and a *Pst*I-*Hae*II fragment from pHFIF-7 into the plasmid pPLa2311. From the resulting plasmid, designated pPLa-HFIF-67-1, a *Bgl*II fragment was excised and ligated into the *Bam*HI site of the β-lactam region of pPLa8 in the sense orientation with respect to the promoter. The known nucleotide sequence around the *Bam* *Bgl*II junction in this plasmid, pPLa-HFIF-67-12, predicts t a polypeptide initiated at the AUG of the β-lactamase part terminate on a double amber stop codon in the 5'-untransla region of the HF-IF gene, 23 nucleotides before the HF initiating AUG (data not shown).

pPLa-HFIF-67-12Δ19 was derived from pPLa-HFIF-67- by deleting a *Hin*dII fragment starting within the β-lactam gene and extending up to three nucleotides before the HF initiating AUG (Fig. 1). From the known nucleotide sequence the β-lactamase gene (as determined on the progeni pBR322)[13] and of the HFIF gene[10], a continuous reading fra starting at the initiating AUG of the β-lactamase gene running up to the terminating UGA of the HF-IF gene predicted. The expected fusion polypeptide consists of 82 am acid residues of the β-lactamase protein, one amino acid co for at the fused *Hin*dII site, and the complete polypept (including the putative signal sequence) specified by the HF gene. The predicted sequence around the junction is: lactamase gene moiety-GUU.AAC. AUG-HFIF gene, wh the GUU triplet codes for amino acid 82 of the β-lactam protein.

Alternatively, a hybrid plasmid with the controllable lamb P_L promoter in the clockwise orientation was constructed. T acceptor plasmid was pPLc24, which contains the P_L promo followed by an *Eco*RI-*Bam*HI fragment (derived from pMS2 [ref. 14]) containing the ribosome binding site and part of t MS2 polymerase gene. The pPLa-HFIF-67-1 *Bgl*II fragme containing the HF-IF gene was inserted into the *Bam*HI site pPLc24, resulting in loss of *Bam*HI and *Bgl*II sensitivity b formation of *Sau*3AI sites at the joints (Fig. 1). In this n plasmid, pPLc-HFIF-67-8, a continuous reading frame starti at the initiating AUG of the MS2 polymerase gene a terminating at the UGA of the HF-IF gene, can be predicted the basis of the known nucleotide sequences of the MS2 pol merase gene[15] and of pHFIF-6 and pHFIF-7 (ref. 10). T expected fusion protein consists of the N-terminal 98 amin acids of the MS2 polymerase moiety, 27 amino acids coded b by sequences between the *Bgl*II site and the initiating AUG the HF-IF gene, followed by the complete HF-IF coding regio

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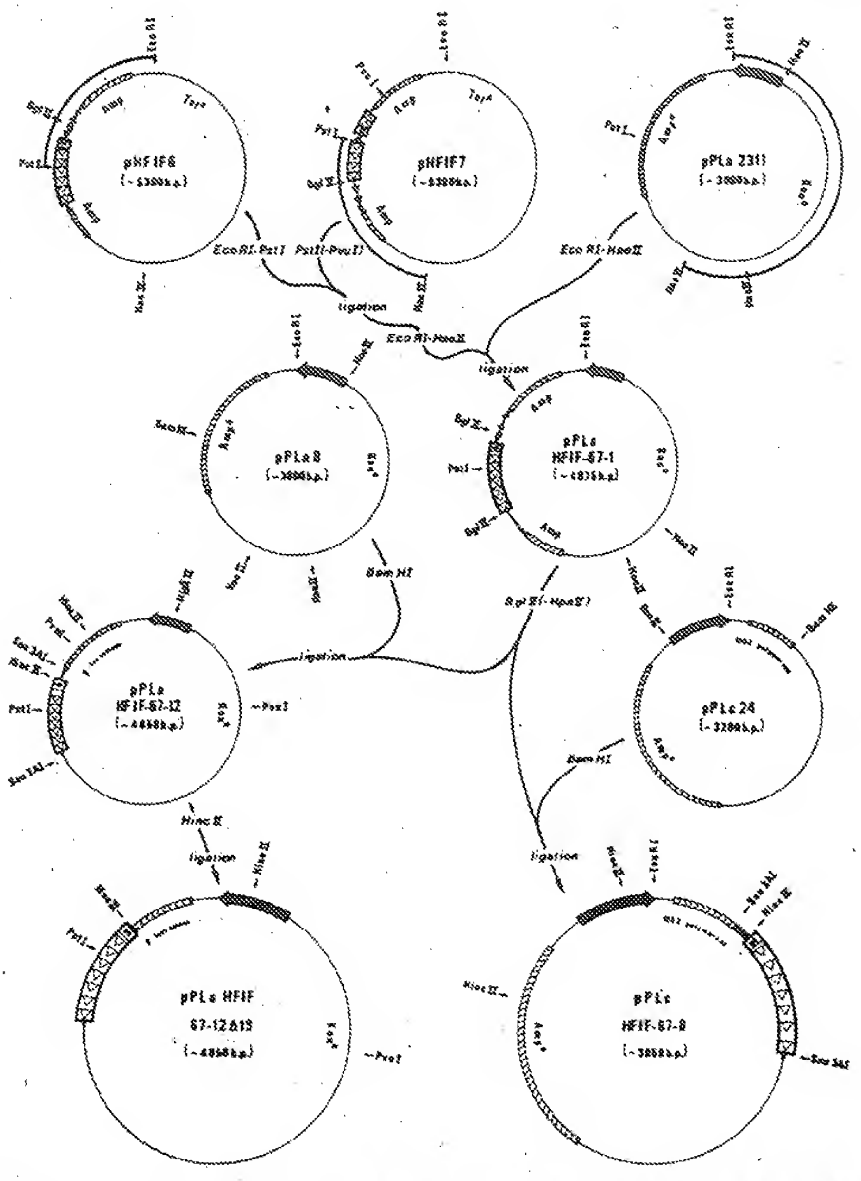

Fig. 1 Schematic outline of the construction of the different plasmids. The HF–IF DNA is indicated by a heavy line, the boxed area being the coding region with the putative signal peptide (S); the triangles indicate the 5'→3' direction of the insert. The heavy arrow indicates the lambda P_L-promoter control element. The ampicillin-resistance region is shown as a shaded area. An interruption of the base line in the insert shows the cross-over region of the inversions of our original HF–IF cDNA-containing plasmids[26]. dA·dT tails are indicated by a wavy line. Amp, β-lactamase region coding for resistance to ampicillin and carbenicillin; Kan, region coding for kanamycin resistance. To construct pPLa-HFIF-67-1, pHFIF-6 DNA was cleaved with *Eco*RI and *Pst*I and ligated to pHFIF-7 which had been cleaved with *Pst*I and *Pvu*I. Following ligation, the mixture was digested with *Eco*RI and *Hin*cII and ligated to pPLa2311 which had been digested with *Eco*RI and partially with *Hin*cII. The segments of the outer circles indicate the fragments retained in the pPLa-HFIF-67-1 construction; only those restriction sites relevant to the constructions are indicated. Transformants were obtained in C600r^-_Km^+_K(λ) with selection for kanamycin resistance and screened for sensitivity to carbenicillin. The structure of a representative plasmid, pPLa-HFIF-67-1, was confirmed by digestions with *Eco*RI, *Pst*I, *Bgl*II and *Hin*cII (= *Hin*dIII). To construct pPLa-HFIF-67-12, the HF–IF gene was excised from pPLa-HFIF-67-1 with *Bgl*II, followed by fragmentation of the remaining part of the plasmid with *Hpa*II, which does not cut the HF–IF gene. The DNA was ligated to pPLa8, which had been opened with *Bam*HI. After ligation, the mixture was redigested with *Bam*HI to eliminate recircularized pPLa8 acceptor plasmids. Transformants were obtained in C600r^-_Km^+_K(λ) by selection for kanamycin resistance. The structure of pPLa-HFIF-67-12 was examined by analysis with *Pst*I and *Hin*cII. To construct pPLa-HFIF-67-12Δ19, pPLa-HFIF-67-12 was partially digested with *Hin*cII and ligated at a low DNA concentration (0.01 μg ml^{-1}). The mixture was then digested with *Xor*II, an isoschizomer of *Pvu*I producing 3'-protruding ends[28], and religated at low DNA concentration, thereby substantially reducing the probability of religation of the original pPLa-HFIF-67-12 plasmids. The structure of pPLa-HFIF-67-12Δ19 was examined by digestion with *Hin*cII and *Pvu*I. To construct pPLc-HFIF-67-8, the DNA of pPLa-HFIF-67-1 was digested with *Bgl*II and ligated into the *Bam*HI-opened pPLc-24 DNA. The ligation mixture was redigested with *Bam*HI to eliminate religated parental pPLc-24 plasmids. Transformants were selected for resistance to carbenicillin. The insertion and orientation of the *Bgl*II HF–IF fragment was established by *Hin*cII digestion. Restriction enzymes (Biolabs, Boehringer or BRL) were used according to the manufacturer's specifications. Ligations were carried out at 22 °C with T4 DNA ligase in Tris-Cl (50 mM, pH 7.6), $MgCl_2$ (5 mM), β-mercaptoethanol (7 mM), ATP (50 μM), (ref. 27). All plasmids were subsequently transferred into *E. coli* M5219 (K12 M72 lac_{am} trp_{am} Sm^R [λCI857ΔHI bio 2521])[24]. Enzymatic reactions were stopped by heating at 65 °C for 10 min.

including the signal peptide. The predicted sequence around the junction is

```
                    -Trp-Asp-Leu-Glu-Phe-Arg-Arg-Gln-Pro-
MS2 polymerase gene society-UGG.GAU.CUU.GAG.UUU.CGC.AGG.CAA.CCU.

Phe-Glu-Ala-Phe-Ala-Leu-Ala-Gln-Gln-Val-Val-Gly-Asp-Thr-Val-Arg-
UUC.GAA.GCC.UUU.GCU.CUG.GCA.CAA.CAG.GUA.GUA.GGC.GAC.ACU.GUU-CGU.

Val-Val-Asn-Met-
GUU.GUC.AAC.AUG-AUIF coding region
```

The first amino acid, tryptophan, corresponds to position 98 of the MS2 polymerase.

All constructed chimaeric plasmids were transformed into *E. coli* C600r$_k^-$m$_k^+$(λ) and, after characterization, transferred into *E. coli* M5219 (ref. 16), allowing the temperature-dependent controlled expression of the lambda P_L (E. Remaut *et al.*, in preparation).

Detection of IF activity in bacterial extracts

Transcription from the P_L promoter on the plasmids can be turned on by shifting the growing culture from 28 to 42 °C. The synthesis of IF-related product(s) was examined by assaying an S100 extract of the bacteria for antiviral activity (Table 1). The cells were lysed either by lysozyme treatment followed by freeze-thawing or by heating in 1% SDS, 1% β-mercaptoethanol, 5 M urea. The extracts of temperature-induced *E. coli* M5219 containing pPLa-HFIF-67-12Δ19 or pPLc-HFIF-67-8 showed a clear antiviral activity, which was reproducibly higher with the latter plasmid. The same non-induced strains as well as induced M5219 containing a reference plasmid (pPLa8) did not show any detectable activity. In M5219 containing pPLa-HFIF-67-12, trace amounts of antiviral activity were occasionally detected after temperature shift (data not shown), presumably due to a rare reinitiation event. The much higher activity obtained after lysis with the SDS, β-mercaptoethanol, urea mixture indicates a possible nonspecific sticking of the antiviral product(s) to bacterial components, for example, cell membranes or nucleic acids. In parallel experiments in which authentic HF–IF was added to a control bacterial extract obtained by lysozyme treatment, only 10–40% of the activity was recovered.

Low but significant amounts of antiviral activity were detected in the supernatant after osmotic shock of induced M5219 transformed with pPLc-HFIF-67-8 (Table 1). When a more severe method of periplasmic extraction was used (that is, spheroplast formation), some activity was also detected with induced M5219 transformed by pPLa-HFIF-67-12Δ19. These results suggest that at least some of the bacterial HF–IF may be secreted into the periplasmic compartment, perhaps concomitantly with the removal of the signal peptide; other explanations, however, cannot be excluded.

Characterization of the bacterial IF activity

The antiviral activity detected in the above-mentioned extracts of induced bacteria was tested for several biological and physical properties characteristic of HF–IF (Table 2). First, the antiviral activity was non-dialysable; after dialysis for 16 h at neutral *p*H the antiviral activity was retained, albeit often at reduced levels (which was also the case for authentic HF–IF preparations). The observed decrease is presumably due to nonspecific sticking to the dialysis membranes, as HF–IF is known to be rather hydrophobic[17], and the unglycosylated bacterial form may be even more so. The antiviral activity could be recovered after precipitation with 67% saturated ammonium sulphate, a concentration known to precipitate HF–IF[18].

When tested for stability at *p*H 2, a common property of fibroblast and leukocyte interferon[1], bacterial HF–IF proved to remain active (Table 2), although again there was often partial loss of activity, but this was also the case with reconstituted authentic HF–IF controls.

The sensitivity of the bacterial HF–IF activity to protease was tested by treating the diluted bacterial extracts with increasing amounts of trypsin. The activity was abolished at the same concentration of trypsin that abolished the activity of authentic HF–IF added to an inactive control lysate.

HF–IF, in contrast to leukocyte interferon, is stable after heating in 1% SDS, 1% β-mercaptoethanol, 5 M urea[19], although we only obtained 10–20% recovery of activity with authentic HF–IF, either alone or in the presence of an inactive bacterial extract (data not shown). The bacterial HF–IF activity remained active in these conditions, as lysis of induced bacteria in this solution resulted in extracts with the highest antiviral titre (Table 1).

The antigenic properties of the *E. coli* IF activity were compared with those of authentic HF–IF. Serial dilutions of goat anti-HF–IF antiserum were incubated with diluted extracts containing bacterial HF–IF activity and with control HF–IF preparations in the presence or absence of an inactive bacterial lysate. The bacterial IF activity was neutralized by the specific antiserum, but some differences were noted in the neutralizing antibody titres for bacterial IF and authentic HF–IF (Table 2). Small differences in neutralization titre were also reported for bacterial leukocyte IF when compared with authentic leukocyte IF[11]. This can be explained by a difference either in antigenicity or in specific IF activity of these bacterial proteins relative to authentic IF.

Table 1 Interferon activity in extracts of *E. coli* M5219 transformed by expression plasmids containing the HF–IF coding sequence

Plasmid	Temperature	Interferon activity (units per ml extract) in S100 extracts after lysis by lysozyme and freeze-thawing (I)	S100 extracts after lysis with SDS, β-mercaptoethanol, urea (II)	Periplasmic fraction: osmotic shock (III)	Periplasmic fraction: spheroplast formation (IV)
pPLa-HFIF-67-12Δ19	28 °C	<3; <2	<30; <100	<2	2
	42 °C	200; 20	200; 2000	<2	10
pPLc-HFIF-67-8	28 °C	<3; <2	<100; <100	<2	<2
	42 °C	200; 50	2,000; 3,000	30	30
pPLa8	42 °C	<3; <2	<100; <100	<2	<2

LB medium (150 ml) was inoculated with 1/500 volume of a fresh seed culture, saturated at 28 °C, and maintained with vigorous shaking at 28 °C until a cell concentration of 2×10^8 ml^{-1} was reached. Induction was by shifting the temperature to 42 °C and incubation of the cultures for 3 h to a final concentration of $4–6 \times 10^8$ cells ml^{-1}. The cells were collected and washed with Tris-HCl (50 mM, *p*H 7.4), NaCl (30 mM) and repelleted. Several different extraction procedures were used: I, the bacterial pellet was resuspended in a final volume (4 ml) with HEPES-NaOH (50 mM, *p*H 7.0), NaCl (30 mM) 3% calf serum, β-mercaptoethanol (3 mM), to which lysozyme (Sigma) was added to 1 mg ml^{-1}. After incubation at 0 °C for 30 min, the suspension was subjected to one or two freeze-thawing cycles. The S100 fraction was prepared by ultracentrifugation at 60,000 r.p.m. for 1 h in a Beckman SW60 Ti rotor. II, The cells were resuspended as in I and lysed in HEPES-NaOH (50 mM, *p*H 7.0), NaCl (30 mM), 3% calf serum, 1% SDS, 1% β-mercaptoethanol, urea (5 M) at 90 °C for 1–2 min. Clearing by ultracentrifugation was as in I. III, Osmotic shock procedure[20]: the bacterial cell pellet was resuspended in 20% sucrose, EDTA (100 mM), Tris-HCl (100 mM, *p*H 7.4), to a cell concentration of 1×10^{10} ml^{-1}. After 10 min at 0 °C, the suspension was centrifuged for 10 min at 10,000 r.p.m. The pellet was resuspended in water to a cell concentration of 1×10^{10} ml^{-1}. After 10 min on ice, the suspension was again cleared for 10 min at 10,000 r.p.m. To this osmotic shock supernatant was added HEPES-NaOH (50 mM, *p*H 7.0), NaCl (30 mM), β-mercaptoethanol (3 mM) and 3% calf serum. IV, Cells were resuspended in 3.6 ml of 0.1 M Tris-HCl, *p*H 8.0, 20% sucrose, to which 0.4 ml of lysozyme (5 mg ml^{-1} in EDTA (20 mM)) was added. After incubation for 30 min at 0 °C, the suspension was centrifuged for 10 min at 10,000 r.p.m. The supernatant was adjusted to 3% calf serum. IF activity was measured by a cytopathic effect (CPE)-inhibition assay on human fibroblasts trisomic for chromosome 21 in microtitre trays. The cells were challenged with vesicular stomatitis virus (Indiana strain) and the CPE was recorded at 24 h. All assays included an internal HF–IF reference which was calibrated against the NIH HF–IF reference G023-902-527. The limit of detection, normally 1 unit ml^{-1}, was often elevated due to toxicity of certain samples (for example, for the samples obtained with method II, the limit of detection was 30–100 units ml^{-1}). The titres are expressed in units ml^{-1}, although it should be noted that they were obtained by 0.5 $\log_{10}$ dilutions.

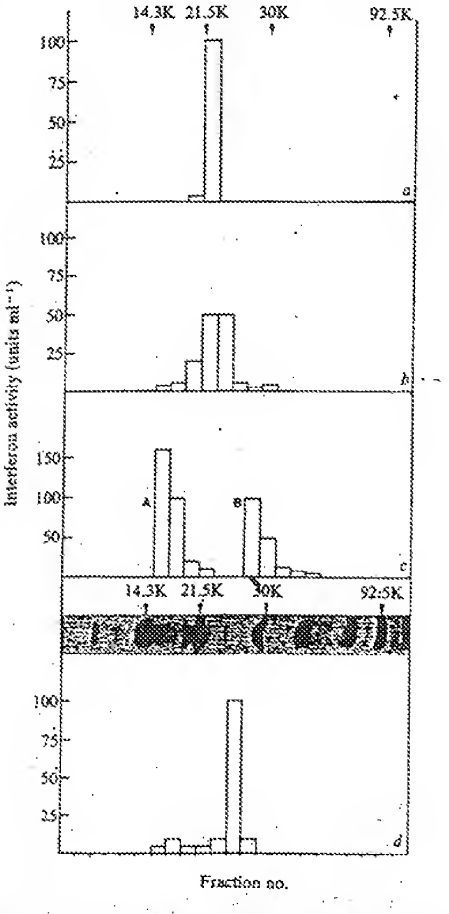

Fig. 2 Polyacrylamide gel patterns of bacterial HF–IF and authentic HF–IF. The following 100-μl samples were loaded on slots of 2 cm width: *a*, authentic HF–IF in Eagle's minimal essential medium, 10% calf serum; *b*, authentic HF–IF in control bacterial extract of M5219/pPLa8 (42 °C); *c*, bacterial extract of M5219/pPLc-HFIF-67-8 (42 °C); *d*, bacterial extract of M5219/pPLa-HFIF-67-12Δ19 (42 °C). ^{14}C-labelled protein markers electrophoresed in an equivalent amount of bacterial extract of M5219/pPLc-HFIF-67-8 (42 °C) are shown as an insertion in *d*. Bacterial extracts were prepared by treatment with SDS, β-mercaptoethanol, and urea (compare with procedure B in legend to Table 1). All samples were boiled for 1 min before electrophoresis, which was run according to Laemmli[28] in 12.5% polyacrylamide gel. IF activity profiles (*a*, *b*, *c*, *d*) were obtained after elution of successive 0.5 cm gel slices for 15 h in 500 μl of 0.5% bovine serum albumin in Tris (0.0125 M), glycine (0.096 M), 0.05% SDS followed by the antiviral IF assay (compare with legend to Table 1). Arrows with molecular weight corresponding to ^{14}C-labelled markers are indicated on top.

HF–IF is largely species specific, exhibiting little (if any) antiviral effect on heterologous cells[1]. Consistent with this property, bacterial IF showed no detectable antiviral protection on cells of monkey, feline, rabbit or mouse origin (Table 3). With respect to the feline cells, bacterial and authentic HF–IF behave differently from human leukocyte interferon.

Further evidence substantiating the presence of active HF–IF in induced *E. coli* extracts was provided by demonstrating the induction of 2′, 5′-oligoadenylate (2–5A) synthetase. Kerr *et al.*[20] first reported that interferon increases the level of this enzyme in susceptible cells. As shown in Table 4, appropriate bacterial extracts were able to enhance the incorporation of [α-^{32}P]ATP in ppp5′A2′p5′A2′p5′A. The 2–5A synthetase-inducing activity of the bacterial extracts was proportional to their antiviral activity. This bacterial IF activity was likewise neutralized by anti-HF–IF antiserum.

Size estimations of bacterial HF–IF

To estimate the molecular weight of the bacterial IF activity in comparison with authentic HF–IF, bacterial extracts were fractionated by polyacrylamide gel electrophoresis in denaturing conditions and the antiviral activity determined for eluates from successive gel slices (Fig. 2). Whilst authentic HF–IF showed a single peak of activity after electrophoresis, the bacterial IF activity appeared in two different peaks. Very accurate molecular weights could not be assigned because of insufficient resolution of the gel; this was mainly due to an overloading effect

Table 1 Characterization of the bacterial HF–IF activity

	Interferon titre (units ml^{-1})	
a, Dialysis at neutral *p*H:	Before	After
M5219/pPLc-HFIF-67-8 (42 °C) (I)	200	200
(II)	1,000	200
(III)	30	20
M5219/pPLa-HFIF-67-12Δ19 (42 °C) (I)	200	20
Control HF–IF in M5219/pPLa8 (42 °C) (I)	200	100
	200	10
b, Precipitation with $(NH_4)_2SO_4$ at 67% saturation:		
M5219/pPLc-HFIF-67-8 (42 °C) (I)	100	100
	100	200
M5219/pPLa-HFIF-67-12Δ19 (42 °C) (I)	100	100
Control HF–IF in M5219/pPLa8 (42 °C) (I)	20	20
	30	20
c, *p*H 2 treatment:		
M5219/pPLc-HFIF-67-8 (42 °C) (I)	100	20
(II)	5	5
M5219/pPLa-HFIF-67-12Δ19 (42 °C) (I)	100	10
Control HF–IF in M5219/pPLa8 (42 °C) (I)	1,000	100
d, Heat treatment in 1% SDS, 1% β-mercaptoethanol, 5 M urea (see Table 1)		

	Inactivating end point concentration (mg ml^{-1})
e, Trypsin digestion:	
M5219/pPLa-HFIF-67-12Δ19 (42 °C) (II)	0.03
M5219/pPLc-HFIF-67-8 (42 °C) (II) (1,000 units ml^{-1})	0.03
Control HF–IF in M5219/pPLa8 (42 °C) (II) (1,000 units ml^{-1})	0.03
M5219/pPLc-HFIF-67-8 (42 °C) (III) (30 units ml^{-1})	0.03
Control HF–IF in M5219/pPLa8 (42 °C) (III) (30 units ml^{-1})	0.03

	Neutralization titre (units ml^{-1})
f, Neutralization by antiserum	
pPLc-HFIF-67-8 (42 °C) (I)	$10^{5.3}$
(II)	$10^{5.8}$
(III)	$10^{4.3}$
pPLa-HFIF-67-12Δ19 (42 °C) (I)	$10^{6.3}$
Control HF–IF in extract of M5219/pPLa8 (42 °C)	$10^{5.7}$
pPLc-HFIF-67-8 (42 °C) (II), elution peak A	$10^{6.4}$; $10^{6.2}$
elution peak B	$10^{5.8}$; $10^{5.7}$
Control HF–IF eluted from polyacrylamide gel	$10^{5.0}$
Control HF–IF	$10^{5.7}$
Control leukocyte IF	<10

g, Anti-viral activity in heterologous cells (see Table 3)
h, 2-5A synthetase induction (see Table 4)

The following experimental methods were used for the characterization: *a*, dialysis at neutral *p*H took place overnight at 4 °C against phosphate buffered saline (PBS). *b*, Two volumes of a saturated $(NH_4)_2SO_4$ solution were added to one volume of extract. After 30 min on ice, the pellet was centrifuged at 12,000*g* for 10 min and redissolved in PBS. *c*, The bacterial extracts were either dialysed for 15 h against glycine-HCl (50 mM, *p*H 2.2), followed by dialysis against PBS for 3 h, or acidified with HCl, followed by neutralization with NaOH. After removal of the precipitate the antiviral activity was determined. *e*, Trypsin digestion was for 1 h at 37 °C with serial dilutions of the enzyme added to the diluted extract. The lowest trypsin concentration that completely abolished the antiviral activity is indicated. *f*, The antibody neutralization assays were carried out essentially as described by Havell *et al.*[32]. About 10 IF units ml^{-1} of the preparations were incubated for 1 h at 37 °C with serial dilutions of goat anti-HF–IF antiserum, after which the residual antiviral activity was determined. Values are presented as neutralizing titres, that is, the highest dilution of antiserum which neutralized the protective effect of IF by 50% multiplied by the interferon titre of the sample assayed. Roman numerals in parentheses refer to the extraction methods described in Table 1.

Table 3 Antiviral protection of bacterial IF activity and authentic interferons

	Interferon activity (units per ml), assayed on					
	Human T-21	Human VGS	Monkey BSC-1	Rabbit primary kidney	Feline lung	Mouse L-929
M5219/pPLc-HFIF-67-8 (42 °C) (II)	3,000	300	<100	<100	<100	ND
(III)	30	<10	<10	<10	<10	ND
M5219/pPLc-HFIF-67-8 (42 °C) (II) elution peak A	2,000	200	<10	<10	<10	<10
M5219/pPLc-HFIF-67-8 (42 °C) (II) elution peak B	2,000	500	<10	<10	<10	<10
Human fibroblast interferon	3,000	300	30	30	<3	<2
Human leukocyte interferon	5,000	500	30	10	1,000	30
Human immune (type II) interferon	3,000	1,000	10	<3	<3	<2
Mouse L-929 interferon	<2	<2	<2	<2	<2	500

The antiviral activity was assayed as described in the legend to Table 1, except that the titres were directly determined from the dilution end points. T-21 are human fibroblasts trisomic for chromosome 21; VGS are normal human diploid fibroblasts[25]. Feline lung cells were obtained from Flow Laboratories (cat. no 0-10907). ND, not determined.

which resulted in a different migration of the proteins, as revealed by internal ^{14}C-labelled protein markers. Both peaks were neutralized to the same extent with anti-HF–IF antiserum (Table 2) and did not show detectable IF activity on heterologous cells (Table 3). The first peak, corresponding to an approximate molecular weight (MW) of 15,000–18,000, may have arisen by haphazard proteolytic cleavage of the fusion protein, or by limited *bona fide* processing at the now internal signal peptide, or by a combination of both processes. As shown previously, the absence of the carbohydrate moieties results in a protein which migrates in polyacrylamide gel to a position of about 4,000 MW below the authentic glycosylated HF–IF[10]. The 15,000–18,000-MW component, clearly present in *E. coli* M5219/pPLc-HFIF-67-8 extract, could also be detected at low but still significant levels in M5219/pPLa-HFIF-67-12Δ19 extracts. The second peak, with an apparent higher molecular weight, could correspond to the fused prokaryotic HF–IF polypeptide, or a slightly processed form. The tentative identification of the slower moving HF–IF activity peak as the fusion protein is strengthened by a different migration of the activity in the extract of M5219/pPLa-HFIF-67-12Δ19 (with a predicted fusion protein of about 28,000 MW) compared to the M5219/pPLc-HFIF-67-8 extract (with a predicted fusion protein of about 33,000 MW) (Fig. 2). The fusion proteins may themselves have some activity or be processed to an active product at the time when the bacterial extract (or gel eluate) is applied onto the human cells for the antiviral assay.

Table 4 2–5A synthetase inducing activity of bacterial lysates and its neutralization by anti-HF–IF antiserum

	^{32}P incorporation (in c.p.m.) into the 2–5A trimer (background subtracted)
a, M5219/pPLc-HFIF-67-8 (42 °C) (III) (30 units ml^{-1})	3,618
Control HF–IF added to M5219/pPLa8 (42 °C) (III) (30 units ml^{-1})	3,695
M5219/pPLa8 (42 °C) (III) (<2 units ml^{-1})	(−1,360)
Control HF–IF (18 units ml^{-1})	1,120
(60 units ml^{-1})	4,338
(180 units ml^{-1})	10,273
(600 units ml^{-1})	21,698
b, Control HF–IF (100 units ml^{-1})	12,468
M5219/pPLa8 (42 °C) (I)	1,011
M5219/pPLa-HFIF-67-12Δ19 (42 °C) (I)	42,193
plus 1 n.u. per ml anti-HF–IF	29,260
plus 100 n.u. per ml anti-HF–IF	727
M5219/pPLc-HFIF-67-8 (42 °C) (I)	17,478
plus 1 n.u. per ml anti-HF–IF	12,115
plus 100 n.u. per ml anti-HF–IF	7,992
plus 10000 n.u. per ml anti-HF–IF	2,517

All samples were diluted sixfold (*a*) or tenfold (*b*) before assay. Antiviral titres (before dilutions) are given in parentheses. Neutralization with goat anti-HF–IF antiserum was at 37 °C for 1 h; n.u. neutralizing units. Roman numerals in parentheses refer to the extraction methods described in Table 1 legend. The obtained values are listed after subtraction of the endogenous background activity: 3,342 c.p.m. in *a* and 2,073 c.p.m. in *b*. The 2–5A synthetase assay was modified from Kimchi *et al.*[26] and Minks *et al.*[27]. Confluent monolayers of HeLa cells in microtitre plates (96 wells) were treated with the diluted bacterial extract or with control HF–IF for 20 h. After cooling and washing with NaCl (140 mM), Tris–HCl (35 mM, *p*H 7.5), the cultures were lysed in 5 μl (*a*) or 10 μl (*b*) of 0.5% NP40, PMSF (1 mM), NaCl (140 mM), Tris–HCl (35 mM *p*H 7.5). After shaking vigorously for 20 min at 0 °C, the lysates were collected and centrifuged for 20 min at 18,000*g*. 3.5 μl of the supernatant was incubated for 2 h at 31 °C in 6 μl of KOAc (100 mM), $Mg(OAc)_2$ (25 mM), HEPES–KOH (10 mM, *p*H 7.4), ATP (5 mM), fructose-1,6-bis-phosphate (4 mM), DTT (1 mM), poly I-C (20 μg ml^{-1}) and 2 μCi of lyophilized [α-^{32}P]ATP (400 Ci mmol^{-1}). The reaction was stopped by heating for 3 min at 95 °C and the samples were treated with 150 units ml^{-1} of calf intestine alkaline phosphatase (Boehringer) at 37 °C for 1 h. After clearing, 1 μl was spotted on PEI-cellulose thin-layer plates and chromatographied in 1 M acetic acid for 2–3 h. The plates were autoradiographed and the incorporation of ^{32}P in the 2–5A trimer was determined.

Conclusion

We have demonstrated the expression of HF–IF activity in *E. coli*. This synthesis depends on the presence of the HF–IF cDNA gene in the appropriate orientation and of the controlled induction of transcription from the P_L promoter. The antiviral activity obtained from *E. coli* is due to the presence of polypeptide(s) which for all physicochemical, biological and immunological characteristics tested closely resembles authentic HF–IF. Polyacrylamide gel electrophoresis resolved the bacterial IF activity into two different size classes; the smaller component presumably resulted from a post-translational cleavage of the fusion proteins.

The HF–IF produced in *E. coli* is still low in titre (about 100 units per 5×10^8 cells ml^{-1}), but undoubtedly this can be improved by better plasmid constructions. Thus, we hope to produce sufficient quantities of bacterial HF–IF to compare its biological and pharmacological properties with those of authentic glycosylated HF–IF and of bacterial leukocyte IF, and perhaps to evaluate its potential clinical applications.

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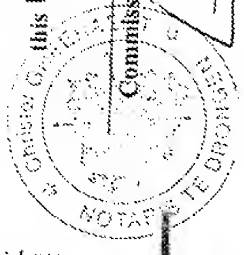
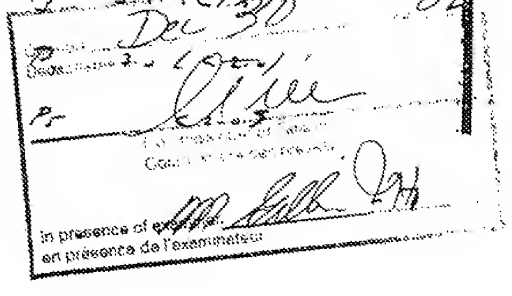
This is EXHIBIT FIERS-27
to
the Affidavit of Walter C. Fiers
sworn before me
this 13 th day of November, 2001

Commissioner for Oath or Notary Public





Gel electrophoresis of whole HFCF
Rabbit - Serum

Gel 1

Lane 1 Empty
Lane 2 1 kb DNA ladder
12.5 µl
5 µl
Lane 3 1 µl of 2.5 mM dNTP
1 µl whole HAI cDNA (~1.2 kb) from HFCF
+ 1 µl 10x Taq buffer
+ 1 µl 10 µM forward primer
+ 1 µl 10 µM reverse primer
+ 5 µl dH2O
Lane 4 2 µl of 2.5 mM dNTP
1 µl whole HAI cDNA (~1.2 kb) from HFCF
+ 1 µl 10x Taq buffer
+ 1 µl 10 µM forward primer
+ 1 µl 10 µM reverse primer
+ 5 µl dH2O
Lane 5 2 µl of 2.5 mM dNTP
1 µl whole HAI cDNA (~1.2 kb) from HFCF
+ 1 µl 10x Taq buffer
+ 1 µl 10 µM forward primer
+ 1 µl 10 µM reverse primer
+ 5 µl dH2O
Lane 6 repeat of Lane 3
Lane 7 repeat of Lane 4
Lane 8 repeat of Lane 5
All other lanes were empty